SYNOPSIS

Synergistic effect and apoptosis induction of flavonoid and acetogenin enriched fractions of *Annona muricata* leaves in Molt-3 cells and Peripheral Blood Lymphocytes

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Introduction

Cancer is the life threatening and dreadful disease characterized by the abnormal proliferation of cells that invade the adjacent tissues and causes the destruction of these tissues; Cancer spreads to distant organs through blood stream and lymphatic vessels (Sultana *et al*., 2014).

The international agency for research on cancer has predicted that India's incidence of cancer will increase from 1 million in 2012 to more than 1.7 million in 2035. This indicates that the death rate because of cancer will also increase from 680000 to 1-2 million in the same period (Varshitha *et al.*, 2015). Cancer is a common disease that claims life about 7-10 million people annually in the world. As a result, cancer remains to be a great medical challenge worldwide (Lu*et al*., 2015).

Leukemia is among hematologic malignance, which have various types and subtypes including Acute Lymphocytic Leukemia (ALL), Acute Myelogenous Leukemia (AML), Chronic Lymphocytic Leukemia (CLL) and Chronic Myelogenous Leukemia (CML). Leukemia is the most common cancer in children (Miladinia *et al.*, 2017).

Acute lymphoblastic leukemia is a malignant cancer of the white blood cells characterized by uncontrolled overproduction and accumulation of lymphoid progenitor cells (Terwilliger *et al.*, 2017). It is most common among children, which compromise 80% of the worldwide acute lymphoblastic leukemia occurrences, although some cases in adults are also seen. It is equally life-threatening in both cases (Baljevic *et al.*, 2016). Leukemia's (>95% of which are acute) constitute the most common diagnostic group of childhood cancers worldwide, and in India (Arora *et al.*, 2016).

The age-adjusted incidence rate in United States is 1.38 per 100,000 populations per year (National Cancer Institute, 2020). Indian incidence varies with region and age-adjusted rates of up to 101.4 per million and 62.3 per million for boys and girls, respectively have been reported (Asthana *et al.*, 2018, Arora *et al.*, 2019). ALL is slightly more common in boys with peak incidence in 2-5 years. T cell ALL constitutes about 15-20% of pediatric ALL; though, in India, a higher proportion of T-ALL (20-50%) has been reported (Arora *et al.*, 2019).

Pediatrics with leukemia is experiencing difference side-effects which induced by disease and its treatments. These side effects are including pain, sleep disturbance, fatigue, anxiety, nausea and vomiting, anxiety, stress, depression, etc. (Miladinia *et al.,* 2017).

Currently cancer treatment and management is a challenge due to complexity of the disease, toxicity of chemotherapy, unaffordability of treatment and severe side effects. Therefore, it is imperative to investigate complementary and alternative medicine for leads and development of anticancer drugs. Drugs from herbal sources are defined as affordable and safe for consumption. The number of medicinal plants used in traditional medicine is large but only few have been investigated for their pharmacological activity. Since ancient times, humans have used traditional medicines for human health care with terrestrial plants occupying a significant therapeutic role (Njagi *et al*., 2016).

Natural products are extremely an important source of medicinal agents. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer based molecular modeling design, none of them can replace the importance of natural products in drug discovery and development (Gavamukulya *et al*., 2014).

Natural products and their molecular frameworks have a long tradition as valuable starting points for medicinal chemistry and drug discovery. Recently, there has been a revitalization of interest in the inclusion of these chemotypes in compound collections for screening and achieving selective target modulation. Natural-product-inspired drug discovery with a focus on recent advances in the design of synthetically tractable small molecules that mimic nature's chemistry. The potential of innovative computational tools in processing structurally complex natural products to predict their macromolecular targets and attempt to forecast the role that natural-product-derived fragments and fragment-like natural products will play in next-generation drug discovery (Rodrigues *et al*., 2016).

One such plant with extensive traditional use is *Annona muricata.* It is a relatively small herbaceous tree located in South America and South East Asia rain forests. The fruits, leaves, stem and roots of *Annona muricata* are known to be rich in flavonoids, isoquinoline alkaloids and annonaceous acetogenins (Moghadamtousi *et al*., 2015, Yajid *et al*., 2018 and Coria-Tellez *et al*., 2018). Flavonoids are polyphenolic compounds, Acetogenins are a class of

polyketide natural product, predominantly present in *Annona muricata* more than 100 *Annonaceous* acetogenins were found to be in the family *Annonaceae* and these are natural antitumor compounds (Yang *et al*., 2015, Roduan *et al*., 2019). These acetogenins demonstrated to be selectively toxic against various types of the cancerous cells without harming healthy cells (Deep *et al*., 2016, Yiallouris *et al*., 2018).

Aim and Objectives

- To perform bioassay guided fractionation, Isolation and characterization of Flavonoid and Acetogenin rich fractions from *Annona muricata* leaves
- To evaluate antioxidant potential of Ethanolic crude extract (ECE), Flavonoid enriched fraction (FEF), Acetogenin enriched fraction (AEF) and combination of FEF and AEF of *Annona muricata* leaves against a team of radicals
- To evaluate anticancer activity of ECE, FEF, AEF and combination of FEF & AEF of *Annona muricata* leaves using Molt-3 and its normal counterpart Peripheral Blood Lymphocytes (PBL)
- To analyze ADME properties and molecular docking of compounds present in AEF with genes involved in apoptotic inductionand targets from leukemic pathway

The study was carried out in 4 different phases for analyzing the combinatorial effect of flavonoid and acetogenin enriched fractions.

Phase – I

Bioassay guided fractionation, isolation and characterization of FEF and AEF from *Annona muricata* leaves

Phase – II

Assessment of antioxidant potential of ECE, FEF, AEF, combination of FEF and AEF of *Annona muricata* leaves

Phase – III

Assessment of anticancer activity of ECE, FEF, AEF, combination of FEF and AEF of *Annona muricata* leaves using Molt -3 and PBL cells

Phase – IV

In silico docking of apoptotic and leukemia targets with AEF phytoconstituents

Results

Phase – I

Qualitative assessment for various phytochemicals present in the *Annona muricata* plant was identified using UV-Visible spectroscopy at the wavelength of 250 to 800nm. The FTIR profile of various solvent forms of *Annona muricata* leaves reveals the presence of various secondary metabolites identified based on the functional groups. The samples showed the presence of alkyl halides, halo compounds, alkenes, anhydrides, secondary alcohols, amines, phenols, alkanes, nitro compounds, alkynes, aldehydes, Isothiocyanates, aliphatic primary amines, alcohols, conjugated aldehydes, alkyl aryl ethers, aromatic amines, aromatic esters, allene and aromatic compounds. The HPTLC and HPLC analysis revealed the presence of Quercetin and Kaempferol like flavonoids in the flavonoid enriched fraction and presence of acetogenins in the acetogenin enriched fraction. The GCMS profile of Ethanolic crude extract of *Annona muricata* leaves extract revealed the presence of 10-Oxodecanoic acid methyl ester, Pentadecanoic acid, Octadecanoic acid, Dodecanoic acid 2-octyl-, α-Gurjunene, α-Humulene, Hexadecatrienoic acid and Nonadecanoic acid; the GCMS analysis of Flavonoid Enriched Fraction revealed the presence of Isoferulic acid, Pentanedioic acid, Myricetin, Apigenin-6-C-glucoside, Luteolin 3′7-di-O-glucoside and Glycitein; the GCMS analysis of Acetogenin Enriched Fraction revealed the presence of α-Terpinene, Cis-Ocimene, α-Muurolene and Germacrene B.

Phase – II

Antioxidant assessment reveals that ethanolic crude extract and all the different fraction form of *Annona muricata* leavespossess radical scavenging activity, radical generation inhibition

activity, reducing power and chelating activity. The radical scavenging results revealed that all the treated groups possess radical quenching ability, among the extracts tested the combination of flavonoid and acetogenin enriched fraction group rendered better radical quenching ability against set of radicals tested namely DPPH, ABTS, hydrogen peroxide and hydroxyl radicals. The radical generation inhibition activity revealed that when increasing the concentration of the fractions there is a better inhibition activity and among the groups tested the combination of flavonoid and acetogenin enriched fraction group rendered maximum inhibition activity against nitric oxide and superoxide radicalsgeneration and this may be due to the combinatorial effect of treatment groups.The reducing power ability results revealed that combination of flavonoid and acetogenin enriched fraction were able to reduce Fe^{3+} ions, the activity increased with an increase in the concentration of the extract and fractions, which indicates the dose-dependent response and maximum activity was found to be at a concentration of 500µg.The Chelating activity results revealed that crude extract and all the fraction shows chelating activity, among the groups tested combination of flavonoid and acetogenin group exhibited significant chelating activity.

Phase – III

The MTT results reveal that extract and fractions were able to induce cell death. Among the treatment groups tested, combination of FEF and AEF rendered good cytotoxicity, followed by FEF, AEF and crude extract at a concentration of 500µg towards the Molt – 3 leukemic cell lines. Since the crude extract and fraction treated groups induce cell death in leukemic cells, it became imperative to assess the ability of treatment groups in PBL, the results revealed that when increasing the concentration of the fractions, there is an increase in the percent viability, which confirms the non-cytotoxic nature of treatment groups in the normal counterpart –PBL and targeting cancerous cells alone. Similar results were observed in Sulphorhodamine B dye reduction assay.

Cell cycle analysis was performed to find out at which stage of cell cycle, the cells were arrested. In the control, the cells were evenly distributed in all the phases of the cell cycle, and uninterrupted indication the normal cell division. Crude extract, FEF and AEF treated cells showed that the cells were arrested at G0-G1 phase of cell cycle whereas in the combination of FEF & AEF treated group showed that a higher proportion of cells were arrested at G0-G1 phase

of the cell cycle, indicating that cells were interrupted during the process of cell division. In case of normal counterpart, peripheral blood lymphocytes,the proportion of cells were evenly distributed in all the phases of the cell cycle, however there is a cell cycle arrest in some of the treated groups with lower proportion of cells when compared to the Molt-3 treated cell lines. This indicates the non-toxic nature of the fractions of *Annona muricata*.

The mechanism of cell death was evaluated using flow cytometry analysis with Annexin V-FITC/ PI. The results showed that, in the control group, a higher percentage of cells were viable compared to the other treatment groups. Administration of FEF & AEF in combination showed apoptotic cell death and more cells were seen in the early and late apoptosis stage. The results obtained for FEF, AEF & crude extracts of *Annona muricata* and combined treatment showed a drastic increase in number of cells undergoing cell death in the early and late apoptotic phase.In the case of PBL treated groups, the number of apoptotic cells were found to be very low, while in some of the treated groups showed apoptotic cell death in early and late apoptosis, with lower proportion of apoptotic cells when compared to treated molt-3 cell lines, which again confirms the non-toxic nature of the fractions in PBL. In the case of combination treated group 93.40% of the cells are in live phase which clearly depicts the protective action in normal cells.

Mitochondrial membrane potential is indicative of cellular changes taking place during cell death.The untreated group has more number of cells existed in the form of J-aggregates. FEF, AEF and Crude extract treated groups showed lesser amount of cells was observed as monomers. In combination of FEF & AEF treated group, significant numbers of cells were found in the form of monomers when compared to FEF, AEF and crude extract treated groups which indicates loss of membrane integrity and mitochondrial membrane potential. In Normal PBL, the untreated and treated group has more number of cells existed in the form of J-aggregates which shows the minimal loss of mitochondrial membrane potential. The results reiterate that *Annona muricata* leaves and the fractions could cause cytotoxicity only in acute lymphoblastic leukemic cells and simultaneously we could observe the peripheral blood lymphocytes are protected by the presence of fractions

Intracellular ROS is a measure of extent of inflammation in the cells. In the control group, there is an increase in the production of ROS level; Prior treatment of molt-3 cells with crude extract and fraction treated groups reduced the production of ROS.Treatment groups

shifted the DCF fluorescence histogram from M2 towards M1. This shift is an indicator of reduction in ROS production. DCF fluorescence intensity was found to be more in the combination of FEF & AEF treated group, when compared to other treated groups, this may be due to the synergistic effect of treated group.In the case of PBL treated groups, the intensity of DCF fluorescence histogram was found to be more in the M1 (Normal cells), however there is partially some of the treated groups shifted to M2 (Cells increase in ROS production), but the DCF fluorescence intensity was found to be less when compared to the molt-3 treated cell lines.

Phase – IV

ADME properties results revealed that α-Terpinene, Cis-Ocimene, α-Muurolene and Germacrene B showed the drug likeness as they have 0 violations, with lipinski's rule of 5.*In silico* docking studies were performed to confirm the interactions of the bioactive compounds ofAcetogenin enriched fraction with the targets from leukemic pathway and genes involved in apoptotic induction. Docking interaction results revealed that Cis-Ocimene has more binding efficiency withNF-Kb, MDM2, Bak, Bcl-2 and Mcl-1 compared to other compounds. Further studies need to be carried out and validated under *in vitro* conditions.

Research findings

A finding of the present study revealed that presence of flavonoids and acetogenins namelyIsoferulic acid, Pentanedioic acid, Myricetin, Apigenin-6-C-glucoside, Luteolin 3′7-di-O-glucoside, Glycitein,α-Terpinene, Cis-Ocimene, α-Muurolene and Germacrene B in the flavonoid and acetogenin enriched fraction of *Annona muricata* leaves. Antioxidant assessment reveals that ethanolic crude extract and all the fraction forms of *Annona muricata* possess radical scavenging activity, radical generation inhibition activity, reducing power and chelating activity. Results of anticancer activity revealed that combination of FEF & AEF treated groups possesses greater cytotoxic effect, and this may be due to the synergistic interaction. In PBL treated group, there is minimal cytotoxic effect, which indicates the non-toxic nature of the *Annona muricata* leaves. Docking results revealed that Cis-Ocimene has more binding efficiency withgenes involved in apoptotic induction and targets from leukemic pathway NF-Kb, MDM2, Bak, Bcl-2 and Mcl-1 compared to other compounds, these results would be lead compounds for the drugs to be focused for *in vitro* and *in vivo* studies in future for exploring therapeutic strategies against leukemia.

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